Thrombin activatable fibrinolysis inhibitor (TAFI) and markers of endothelial cell injury in dialyzed patients with diabetic nephropathy

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Summary
Patients dialyzed due to diabetic nephropathy are at a higher risk of death due to cardiovascular complications than dialyzed non-diabetic patients. Disturbances in hemostasis may play a role in the vascular complications of diabetes mellitus. It has been postulated that TAFI-thrombin activatable fibrinolysis inhibitor, which couples two opposite systems: coagulation and fibrinolysis, may be involved in the mechanism of vascular endothelial damage in diabetic patients. We assessed: TAFI and TAFIa, markers of ongoing coagulation: thrombin-antithrombin complexes, prothrombin fragments 1+2, a marker of ongoing fibrinolysis: plasmin-antiplasmin complexes in diabetic and non-diabetic patients on hemodialyses-HD, peritoneal dialyses-CAPD, patients with chronic renal failure with and without diabetic nephropathy on conservative treatment. Both groups of dialyzed diabetic patients have a higher concentration of markers of ongoing coagulation and TAFI activity when compared to dialyzed non-diabetic patients. Linear regression analysis showed that TAFI concentration was directly related to albumin in HD and CAPD patients without diabetic nephropathy, whereas TAFIa correlated with triglycerides, fibrinogen and leukocytes count in this group. When evaluated separately (HD, CAPD), significant correlations between TAFIa and triglycerides and fibrinogen were found only in diabetic CAPD patients. Multivariate analysis showed no correlation between TAFI and other parameters studied. In conclusion, elevated circulating TAFI and TAFIa might be a new link in the pathogenesis of impaired fibrinolysis in diabetic nephropathy, and thus atherosclerosis progression, particularly in CAPD patients. Hypercoagulable state observed in diabetic patients on conservative treatment and maintained on dialyses may contribute to the higher cardiovascular mortality in this population. In these patients there is also evidence of endothelial injury, and probably secondary activation of the coagulation cascade.

Keywords
TAFI, diabetic nephropathy, hemodialyses, peritoneal dialyses, endothelium

Introduction
Pathogenesis of hemostatic disturbances and thrombotic complications in diabetic nephropathy are not fully understood and despite many years of research still remain an unresolved issue. Thromboembolic complications are a major threat to diabetic patients (1-3). Common predisposing factors are: hypofibrinolysis and enhanced coagulation in diabetic patients (4-10). Disturbances in hemostasis are also common in kidney diseases (11). Their occurrence and severity correlate quite well with the progressive loss of renal function to end-stage renal disease. The principal cause of these abnormalities is the uremic state which, as a rule, is at least partially reversible with the institution of adequate renal replacement therapy.
Continuous ambulatory peritoneal dialysis (CAPD) with its tendency towards hypoalbuminemia and hypertriglyceridemia appears to mimic the metabolic abnormalities that account for the hypercoagulability in the nephrotic syndrome (12). Hemodialysis with extracorporeal circulation and exposure to heparin may also contribute to the prothrombotic state due to recurrent platelet stimulation leading to their hyperaggregability, reduced levels of heparin cofactor II and increased concentrations of coagulation factors (12-14). In dialyzed diabetic patients, hemostatic disturbances associated with hyperglycemic state are superimposed on the abnormalities in coagulation and fibrinolysis due to uremic state and renal replacement therapy. In dialyzed diabetic patients, high incidence of premature micro- and macroangiopathy contributed to the increased mortality and morbidity when compared to dialyzed non-diabetic patients. We have reported that patients on CAPD showed evidence of a higher degree of hypercoagulation than HD subjects (15). We observed also that non-dialyzed patients with diabetic nephropathy exhibited disturbances in hemostasis when compared to diabetic patients without nephropathy and to the healthy volunteers (8, 9).

Thrombin activatable fibrinolysis inhibitor (TAFI) is a recently discovered glycoprotein which couples two systems distinct in function coagulation and fibrinolysis (16). It is present in plasma as a proenzyme and can be activated by thrombin to TAFIa in a manner potentiated by thrombomodulin to yield a thermally unstable enzyme capable of inhibiting fibrinolysis (17). TAFIa removes COOH-terminal lysine and arginine residues from fibrin, impairing formation of t-PA, plasminogen, and fibrin complex, making plasmin generation less effective (18). At high concentrations, TAFI is a plasmin inhibitor too (18). In a very recent study, Yano et al. (19) reported that increased level of TAFI may be involved in the vascular endothelial damage in patients with type 2 diabetes mellitus.

As far as we know, there is no data on TAFI in dialyzed diabetic patients, therefore the aim of the study was to evaluate TAFI concentration and activity, thrombin activity, thrombin and plasmin generation markers in this population relative to the method of renal replacement therapy: peritoneal dialyses vs hemodialyses. Due to the fact that endothelial cell damage is invariably associated with clinical conditions such as thrombosis, hypertension, renal failure and atherosclerosis (20), thrombomodulin, a marker of endothelial cell injury, was also studied.

Materials and methods

The study was performed on 2 groups of clinically stable dialyzed patients: group I - 79 chronically hemodialyzed patients and group II - 34 patients maintained on chronic ambulatory peritoneal dialysis-CAPD and 2 groups of patients with chronic renal failure-CRF: 14 patients with non-diabetic nephropathy and 10 patients with diabetic nephropathy. Inclusion criteria were: a stable clinical state, no thrombosis or inflammation, (C-reactive protein within normal range), absence of cardiovascular complications (including uncontrolled hypertension), no oral contraception in women of child-bearing age, stable and no more than twice of the normal AspAT and AlAT activities. None of the patients investigated had received blood transfusions for at least 1.5 months and no drugs known to affect hemostasis were administered for at least 2 weeks prior the study.

The HD-non DM group consisted of 51 patients without diabetic nephropathy (18F, 33M, age range 43-78 years) and the HD- DM group comprised 28 subjects (12F, 16M, age range 19-79 years). The causes of renal failure among HD patients without diabetic nephropathy varied between chronic glomerulonephritis (n=20), chronic interstitial nephritis (n=16), polycystic kidney disease (n= 4) and other or unknown causes (n= 6). All the diabetics were treated with subcutaneous insulin. 54 patients (14 diabetics) were treated with recombinant human erythropoietin (Epred, Janssen-Cilag, Switzerland), mean dose was 91±16 U/kg/week. All the patients had required regular hemodialyses for 4-5 hours a day, 3 times a week. Blood flow was usually 180-230 ml/min with a dialysate flow rate of 500 ml/min. Ultrafiltration was varied according to patient’s actual weight. Among all HD patients, 49 subjects were dialyzed on polysulphone membranes ( Fresenius, Bad Homburg, Germany) and 30 on cuprophone membranes (Gambug, Nipro, Braun or Terumo dialyzers). 16 patients were dialyzed with acetate dialysates and 63 with bicarbonate dialysates. In HD subjects, blood was drawn in the morning between 8.00 and 9.00 am. to avoid circadian variations (21) before the onset of dialysis session (and heparin administration- unfractionated heparin in 10 subjects, LWMH-enoxaparine or fraxiparine in 69 patients, all diabetics were given enoxaparine) and after hemodiagnosis from the arterial line of hemodialysis system immediately before discontinuation of the extracorporeal circulation.

The PD-DM group consisted of 18 patients (8F, 10M, age range 42-71 years) and the PD-non DM group comprised 24 subjects (7F, 17M, age range 22-71 years). In CAPD patients without diabetic nephropathy renal failure was due to glomerulonephritis (n= 6), chronic interstitial nephritis (n=5), polycystic kidney disease (n=3) and other or unknown causes (n= 3). All the diabetics were treated with subcutaneous insulin. 22 patients (6 diabetics) were treated with recombinant human erythropoietin (Neo-Recormon, Roche, Switzerland), mean dose was 69±18 U/kg/week. A 24-hour dialysate was collected and blood samples were drawn in the morning when subjects, receiving their normal diet, appeared for routine office assessment of dialysis therapy after an overnight oral fast. All the CAPD patients were performing four 2 l exchanges. They were using Baxter Twin Bag system or Fresenius Andy Plus system. Dwell times were generally 4-6 hours during the day and 8 hours overnight. The osmotic pressure of CAPD fluid was adjusted in accordance with the extent of ultrafiltration in each patient (mean ultrafiltration was 8989 (618 ml/24h).
14 patients with chronic renal failure were on conservative treatment (mean proteinuria 1.43 (1.08g/d, serum creatinine 5.25±2.61 mg/dL). All of them were biopsied and histopathological diagnosis was established as follows: IgA nephropathy in 6 cases, membrano-proliferative glomerulonephritis in 2 cases, membranous nephropathy in 2 cases, focal segmental glomerulosclerosis in 2 cases, submicroscopic glomerulonephritis in 1 case. Biopsy was not diagnostic in 1 case. During the study none of the patients received prednisone, anticoagulants or cytotoxic drugs. Patients with diabetic nephropathy due to type 2 diabetes mellitus were also included in the study (proteinuria 1.52±1.2 g/d, HbA1c= 8.9±9 %, 4.50±0.38 mg/dL). All of them were treated with subcutaneous insulin.

Twenty six age-and sex-matched healthy volunteers served as a control group to obtain normal ranges for studied hemostatic parameters. The patients’ height and weight were recorded for all groups. All the patients were informed about the aim of the study and gave their consent. The study was approved by Local Ethic Committee.

Blood was drawn in the morning between 8.00 and 9.00 am. to avoid circadian variations (21) after an overnight fast. Blood was taken without stasis. Venous blood samples were collected into 3.8% sodium citrate in 9:1 volume ratio. The blood was centrifuged at 1900 g for 20 min at room temperature to yield platelet poor plasma (PPP). Samples were aliquotted and stored at -40°C before assay.

We studied TAFI concentration (TAFI-EIA, Affinity Biologicals Inc., Canada) and TAFIa (Actichrome TAFIa, American Diagnostica, USA). A single standard curve was constructed with the use of standard plasma (BioMerieux, France). All results were expressed as a percentage of standard plasma TAFI concentration. We also evaluated thrombin activity (thrombin-antithrombin complexes- TAT, Enzygnost TAT micro, Dade Behring, Germany, prothrombin fragments 1+2, Enzygnost F1+2 micro, Dade Behring, Germany)- TAFI activator, thrombomodulin (TM, IMUBIND Thrombomodulin ELISA Kit, American Diagnostica Inc., USA)– catalyst of TAFI activation and a marker of endothelial cell injury, and the degree of plasmin generation.

![Table 1: Clinical characteristics of the chronic renal failure patients (CRF non DM), patients with diabetic nephropathy on conservative treatment (CRF DM), hemodialyzed patients without diabetic nephropathy (HD Non DM) and with diabetic nephropathy (HD DM), peritoneally dialyzed patients without diabetic nephropathy (CAPD Non DM) and with diabetic nephropathy (CAPD DM)](image-url)
(plasmin-antiplasmin complexes PAP, Enzygnost PAP micro, Dade Behring, Germany) by the use of commercially available kits. The interassay and intrassay coefficients of variability for each kit were less than 10%. Hemoglobin, erythrocyte count, platelet count, fibrinogen, total protein, cholesterol, triglycerides, albumin concentration, fasting glucose, prothrombin time, activated partial thromboplastin time were measured by standard laboratory methods. These parameters were not assessed in healthy volunteers. Euglobulin clot lysis time, which reflects overall fibrinolytic activity was measured according to Kowalski, et al. (22). To make euglobulin lysis time-independent of fibrinogen concentration, we calculated fibrinolytic activity index (FAI = fibrinogen divided by euglobulin lysis time).

Data given were analyzed using Statistica 5.0. computer software. If possible, data were logarithmically transformed to achieve normal distribution. Normality of variable distribution was tested using Shapiro-Wilk W-test. Measurements normally distributed are reported as mean ± SD, non-normally distributed data are expressed as a median and minimal-maximal value. Analysis of variance (ANOVA) (with post hoc Tukey test for unequal groups) or Kruskall-Wallis ANOVA (the difference between the mean of two variables was calculated by Mann-Whitney U test) were used in statistical analysis to compare differences between groups with p<0.05 considered statistically significant, when appropriate. Linear regression analysis employed Pearson or Spearman coefficients as appropriate.

**Results**

Clinical characteristics of the groups studied as well as some biochemical data are given in Table 1. ECLT was significantly prolonged in all the groups of patients studied when compared to the healthy subjects (232.1 ± 50.9 min, p<0.001) and FAI was significantly lower in all groups of patients, except non diabetic CAPD subjects over controls (1.31 ± 0.45, p<0.05). Other parameters presented in Table 1 were not assessed in the control group. CRF patients with and without diabetes mellitus have higher Hb, erythrocyte count than dialyzed patients. Patients with CRF have higher cholesterol levels when compared with relevant groups of hemodialyzed patients. Patients with diabetic nephropathy have prolonged ECLT and lower FAI than CRF patients without diabetic nephropathy. In both groups of patients on CAPD serum cholesterol, triglycerides, and fibrinogen were significantly higher than in the relevant groups of hemodialyzed patients. ECLT was significantly prolonged in diabetic CAPD patients when compared to non-diabetic CAPD subjects. FAI was significantly higher in non-diabetic in comparison to diabetic CAPD patients. Moreover, FAI was significantly higher in non-diabetic CAPD patients when compared to non-diabetic HD subjects. Markers of thrombin generation (TAT complexes, prothrombin fragments 1+2) were significantly higher in dialyzed diabetic patients relative to dialyzed patients without diabetic nephropathy (Fig. 1). All these parameters were significantly higher in dialyzed patients when compared to the control group (all p values less than 0.001). F1+2 in CRF patients with diabetic nephropathy was significantly higher than in CRF patients without diabetic nephropathy and lower than in diabetic CAPD patients. PAP concentrations did not differ significantly between all groups of patients studied. TAFI and TAFI in diabetic CAPD patients were significantly higher when compared to non-diabetic CAPD subjects (Fig. 2). In HD, only TAFI was significantly higher in diabetic HD patients relative to non-diabetic HD subjects. TAFI concentration in non-diabetic hemodialyzed and peritoneally dialyzed patients, TAFI in non-diabetic hemodialyzed patients did not differ from the control group. TAFI and TAFI in diabetic CAPD patients, TAFI in diabetic HD patients, non-diabetic CAPD patients, TAFI concentration in diabetic HD patients significantly higher in dialyzed patients when compared to the control group. TAFI and TAFI were significantly higher in patients with diabetic nephropathy when compared to CRF patients and lower than compared to diabetic CAPD patients.

Linear regression analysis showed that TAFI concentration was directly related to albumin in non-diabetic dialyzed patients (both HD and CAPD), (r=0.44, p<0.05), time on renal replacement therapy (r=0.32, p<0.05) and Kt/V (r=0.31, p<0.05). TAFI correlated with triglycerides (r=0.43, p<0.05), fibrinogen (r=0.43, p<0.05) and leukocytes count (r=0.44, p<0.05) in dialyzed non-diabetics. However, evaluation of these correlation

![Figure 1: Markers of thrombin generation (F1+2 - prothrombin fragments 1+2, TAT-thrombin-antithrombin complexes) and a marker of plasmin generation (PAP-plasmin-antiplasmin complexes) in patients with and without diabetic nephropathy maintained on hemodialyses (HD), peritoneal dialyses (PD) or conservative treatment (CRF). F1+2 and TAT complexes were significantly higher in dialyzed and CRF patients when compared to the control group (all p values less than 0.001, p not shown on the figure).](image-url)
separately in the all groups of dialyzed patients (HD, CAPD) reveal lack of correlations between TAFI concentration and albumin, but significant correlation between TAFIa and triglycerides ($r=0.64$, $p<0.05$) and fibrinogen ($r=0.60$, $p<0.05$) only in diabetic CAPD patients. In non-diabetic dialyzed patients (both HD and CAPD), TAFI concentration was related to time on renal replacement therapy ($r=0.41$, $p<0.05$) and $Kt/v$ ($r=0.39$, $p<0.05$). In the healthy volunteers, TAFIa correlated positively only with TAT ($r=0.44$, $p<0.05$) and thrombomodulin ($r=0.55$, $p<0.01$) whereas TAFI concentration was inversely related to PAP ($p=-0.41$, $p<0.05$). Multivariate analysis showed no correlation between TAFI, TAFIa and other parameters studied in dialyzed patients, CRF patients as well as in the control group.

**Discussion**

To our knowledge, this is the first report on TAFI in dialyzed patients with diabetic nephropathy. The acronym TAFI implies that its pathophysiological role is the regulation of fibrinolysis. TAFIa exhibits carboxypeptidase B-like specificity, which inhibits fibrinolysis by catalyzing the removal of $C$-terminal lysines of fibrin, thus impairing plasminogen binding. Thus, TAFI and its activation by thrombin-thrombomodulin and plasmin constitute an antifibrinolytic pathway analogous to the anticoagulant protein $C$ pathway. It can be expected that changes in TAFI concentration are involved in disturbances in hemostasis: bleeding or thrombotic syndromes. The demonstration that TAFI activation requires intact intrinsic coagulation pathway (23) suggests that bleeding due to intrinsic coagulation defect may, in part, result from abnormal TAFI activation. On the other hand, TAFI is required for the profibrinolytic effect of activated protein $C$ (23). Thus, excessive TAFI activation may be an additional contributing factor to thrombosis, a common complication of peritoneal dialysis. Thrombomodulin, in adequate concentration (in soluble, as well as in cellular form), plays a key role in the activation of TAFI by thrombin (17). In our study we found that TAFI concentration and activity in dialyzed patients with diabetic nephropathy was significantly higher that in the relevant groups of dialyzed patients without diabetic nephropathy. Previously, we have reported that TAFI concentrations were significantly higher in CAPD (24) as well as in kidney transplant recipients (25), two populations of kidney patients with decreased fibrinolytic activity and a hypercoagulable state. We reported that in CAPD patients there is an evidence of higher degree of hypercoagulation than in HD subjects (15). Thus, we supposed that TAFI might play a role in disturbances in fibrinolytic system in dialyzed patients due to diabetic nephropathy.

Hori, et al. (26) have shown that increased circulating level of TAFI may be an important causative factor of hypofibrinolysis in patients with type 2 diabetes. Antigen level of TAFI (142.1±20.6%), measured by a commercially available kit from Kordia Laboratory Supplies, was similar to values obtained in our patients with diabetic nephropathy on conservative treatment (144.1±31.54%) using a commercially available kit from Affinity Biologicals. However, Hori et al. (26) did not describe patients in regard to renal function and/or proteinuria. In one more paper from this group (27), increased TAFI levels were again described in diabetic patients (30 with normoalbuminuria and 10 with microalbuminuria). Moreover, TAFI correlated significantly with APC-PCI complex, a marker of ongoing protein $C$ activation. It may suggest that activated protein $C$ may promote fibrinolysis in diabetic patients by modulating action of TAFI. Hori et al. (26) and Yano et al. (27) reported increased TAT levels in diabetic patients as well as decreased ration between plasma levels of D-dimers and TAT, an index of fibrinolytic activity. Their findings corroborate with our data about hypercoagulable state and hypofibrinolysis in diabetic patients on conservative treatment and maintained on dialysis. In a very recent study, Yano et al. (19) observed that circulating TAFI level is significantly increased in diabetic patients with microalbuminuria and with serum creatinine less than 100µmol/L. The weak correlation between TAFI and thrombomodulin in the multivariate analysis found in this study suggests that, apart from vascular endothelial cell injury, other factors may be involved in the increased circulating TAFI levels in
diabetic patients. In our study, higher TAFI and TAFIa in diabetic dialyzed patients may be due to the fact that proportionally less diabetic patients received rHuEPO and had higher platelet count. We have reported that a long-term rHuEPO therapy resulted in TAFI decrease in CAPD patients (28). Very recently, Mosnier et al. (29) identified TAFI in human platelets. The concentration of TAFI in platelets represents a very small percentage of the total amount of plasma TAFI, whereas the concentration of TAFI in platelet is in the same order of magnitude compared with plasma TAFI. Since diabetic platelet are hyperactive (21), diabetes is a hypercoagulable state and platelets are concentrated within the fibrin clot, local concentration of TAFI could approach significant levels. In our study we observed lack of correlation between TAFI, TAFIa and thrombomodulin, but thrombomodulin correlated positively with time on dialyses and negatively with Kt/V-a marker of dialysis adequacy. In the control group thrombomodulin correlated with TAFI concentration. Thrombomodulin concentration is closely related to kidney function (30), and elevation of this marker of endothelial cell injury, is a common finding in uremia (11, 15). Thrombomodulin concentration between diabetic and non-diabetic dialyzed and conservatively treated patients did not significantly differ in our study as TAFI and TAFIa did.

In the control group, PAP correlated with TAFI, which corroborates with the previous findings of Mosnier, et al. (31) who reported that TAFI in concentration-dependent manner prolonged clot lysis at least in vitro. Moreover, infusion of tPA with TAFIa inhibitor enhanced thrombolysis or markedly reduced the amount of tPA needed to achieve the same efficacy. A report by van Tilburg (32) showed that high TAFI was a risk factor for deep vein thrombosis, therefore inhibition of TAFIa might provide enhanced fibrin-specific thrombolysis. In an experimental study, TAFIa inhibition reduced mortality caused by thrombin-induced thromboembolism by 50% (33). Lack of correlation between TAFI and PAP in dialyzed patients (both diabetic and non-diabetic) may be due to the fact that TAFI complexes are not a sensitive marker of plasmin generation in dialyzed subjects. Nakamura, et al. (34) reported that HD patients exhibited high PAP levels but low plasmin activity. Moreover, Tomura et al. (35) showed lack of correlation between PAP and tPA and PAI in HD. In dialyzed non-diabetic patients, TAFI activity correlated with leukocyte count. It may be due to the fact that even sub-clinical inflammation, common in uremia, caused a rise in leukocyte count (however, still within normal ranges) but also an activation of coagulation (36), resulting in enhanced thrombin generation. It may lead to TAFI activation and impairment of fibrinolysis. In diabetic dialyzed patients, F1+2 and TAT were significantly higher relative to dialyzed non-diabetic patients. It appeared that in dialyzed diabetic patients an enhanced thrombin generation took place. It may explain an increased TAFI activation since thrombin, which is produced in excess in dialyzed diabetics, is a natural activator of TAFI. Thus, what is the reason(s) of enhanced activation of coagulation? Endothelial cell injury is the probable cause due to uremic toxins retention, dyslipidemia, hypertension and secondary hyperparathyroidism, as well as increased levels of cytokines such as IL-1 and TNFα. Dyslipidemia and hypertension were more prevalent in dialyzed diabetics than in non-diabetic patients on dialyses. However, in dialyzed patients the question arises as to whether activation of fibrinolysis is primary or secondary. According to Lane, at al. (37) and others (38-42), in hemodialyzed and peritoneally dialyzed patients, hyperfibrinolysis is secondary to activation of coagulation cascade. At the same time, overall fibrinolytic activity is impaired (15, 43, 44). Opatrny, et al. (44) reported a fibrinolysis defect manifesting after standard fibrinolytic stimulus (DDAVP-1-deamino-8-D-arginine vasopressin) by an insufficient decrease in PAI-1 concentration in patients with type 2 diabetes mellitus maintained on chronic hemodialysis. On the other hand, Babazono et al. (45) compared coagulation and fibrinolysis in 23 diabetic patients on long-term CAPD with hemodialyzed diabetic patients. They found that CAPD patients had higher fibrinogen, von Willebrand factor, relative to HD patients as well as higher serum lipids. They concluded that CAPD was associated with more atherogenic lipid profile than were on hemodialyses and with hypercoagulable state but not with decreased fibrinolysis state.

Kluft, et al. (46) have found elevated TAFI antigen level in patients with unstable angina pectoris. Since impairment of fibrinolysis and hypercoagulability, thromboembolic complications, as well as the increased incidence in coronary heart disease (47) are common in diabetes mellitus, inhibition of TAFIa in diabetic patients may be of a particular clinical importance.

In conclusion, elevated circulating TAFI and TAFIa might be a new link in the pathogenesis of impaired fibrinolysis in diabetic nephropathy, predisposing to the thrombosis and atherosclerosis progression, particularly in CAPD patients. In these patients there is also evidence of endothelial injury and probably secondary activation of the coagulation cascade.

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